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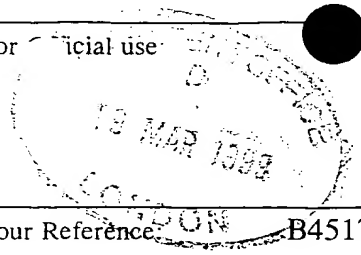

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Form 1/77 **Patents Act 1977**

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1 Please give the title of the invention VACCINE

*❷ Do not give trading styles, for example, 'Trading as XYZ company', nationality or former names, for example, 'formerly (known as) ABC Ltd' as these are not required.*

## ❷ Applicant's details

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Agent's address SmithKline Beecham
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4. Agent's or applicant's reference number *(if applicable)* MJWD/B45175

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5. Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

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❼

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Continuation sheets for this Patents Form 1/77 [ – ]

Claim(s) 3 Description 26

Abstract [ ] Drawing(s) [ ]

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Patents Form 7/77 - Statement of Inventorship and Right to Grant [ – ]

Patents Form 9/77 - Preliminary Examination Report [ – ]

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# VACCINES

The present invention relates to polysaccharide antigen – Protein D conjugates, their use as immunogenic compositions, their manufacture and the use of such conjugates in medicines.

Polysaccharide antigen based vaccines are well known in the art, and four that have been licensed for human use include the Vi polysaccharide of *Salmonella typhi*, the PRP polysaccharide from *Haemophilus influenzae*, the tetravalent meningococcal vaccine composed of serotypes A, C, W135 and Y, and the 23-Valent pneumococcal vaccine composed of the polysaccharides corresponding to serotypes 1, 2, 3,4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33. .

The latter three vaccines confer protection against bacteria causing respiratory infections resulting in severe morbidity and mortality in infants, yet these vaccines have not been licensed for use in children less than two years of age because they are poorly immunogenic in this age group.

The licensed polysaccharide vaccines listed above have different demonstrated clinical efficacy. The Vi polysaccharide vaccine has been estimated to have an efficacy between 55% and 77% in preventing culture confirmed typhoid fever (Plotkin and Cam, Arch Intern Med 155: 2293-99). The meningococcal C polysaccharide vaccine was shown to have an efficacy of 79% under epidemic conditions (De Wals P, et al. Bull World Health Organ. 74: 407-411). The 23-valent pneumococcal vaccine has shown a wide variation in clinical efficacy, from 0% to 81% (Fedson et al. Arch Intern Med. 154: 2531-2535). The efficacy appears to be related to the risk group that is being immunised, such as the elderly, Hodgkin's disease, splenectomy, sickle cell disease and agammaglobulinemics (Fine et al Arch Intern Med. 154:2666-2677), and also to the disease manifestation. Pneumococcal pneumonia and Otitis media are diseases, which do not have demonstrated protection by the 23-valent vaccine. It is generally accepted that the protective efficacy of the pneumococcal vaccine is more or less related to the concentration of antibody induced upon vaccination; indeed, the 23 polysaccharides were accepted for licensure solely upon the immunogenicity of each component

polysaccharide (Ed. Williams et al. New York Academy of Sciences 1995 pp. 241-249).

Amongst the problems associated with the polysaccharide approach to vaccination, is the fact that polysaccharides *per se* are poor immunogens. Strategies which have been designed to overcome this lack of immunogenicity include the linking of the polysaccharide to large highly immunogenic protein carriers, which provide bystander T-cell help.

Examples of these highly immunogenic carriers which are currently commonly used for the production of polysaccharide immunogens include the Diphtheria and Tetanus toxoids (DT and TT respectively), Keyhole Limpet Haemocyanin (KLH), and the purified protein derivative of Tuberculin (PPD). A number of problems are associated with each of these commonly used carriers, including both problems in production of GMP constructs and also in immunological characteristics of the constructs. The present invention provides a new carrier for use in the preparation of peptide-based immunogen constructs, that does not suffer from the aforementioned disadvantages.

However, despite the common use of these carriers and their success in the induction of anti polysaccharide antibody responses they are associated with several drawbacks. For example, it is known that antigen specific immune responses may be suppressed by the presence of preexisting antibodies directed against the carrier, in this case Tetanus toxin (Di John *et al*; Lancet, December 16, 1989). In the population at large, a very high percentage of people will have pre-existing immunity to both DT and TT as people are routinely vaccinated with these antigens. In the UK for example 95% of children receive the DTP vaccine comprising both DT and TT. Other authors have described the problem of epitope suppression to peptide vaccines in animal models (Sad *et al*, Immunology, 1991; 74:223-227; Schutze *et al*, J. Immunol. 135: 4, 1985; 2319-2322).

In addition, for vaccine which require regular boosting the use of highly immunogenic carriers such as TT and DT are likely to suppress the polysaccharide antibody response after several injections. These multiple vaccinations may also be accompanied by undesirable reactions such as delayed type hyperresponsiveness (DTH).

KLH is known as potent immunogen and has already been used as a carrier for IgE peptides in human clinical trials. However, some adverse reactions (DTH-like reactions or IgE sensitisation) as well as antibody responses against antibody have been observed.

The selection of a carrier protein, therefore, for a polysaccharide based vaccine will require a balance between the necessity to use a carrier working in all patients (broad MHC recognition) and the induction of high levels of anti-polysaccharide antibody responses and of low antibody response against the carrier.

The carriers used previously for polysaccharide based vaccines, therefore have many disadvantages.

The present invention provides a protein D from *Haemophilus influenzae*, or fragments thereof, as a carrier for polysaccharide based immunogenic composition, including vaccines. Fragments suitable for use include fragments encompassing T-helper epitopes. In particular protein D fragment will preferably contain the N-terminal 1/3 of the protein.

Protein D is an IgD-binding protein from *Haemophilus influenzae* (EP 0 594 610 B1) and is a potential immunogen. The inventior, Forsgren, suggests that it may be fused to other genes and expressed to form fusion proteins.

Polysaccharides to be conjugated to Protein D contemplated by the present invention include, but are not limited to the Vi polysaccharide antigen against *Salmonella typhi*, meningococcal polysaccharides including type A, C, W135 and Y, the polysaccharide and modified polysaccharides of group B meningococcus, polysaccharides from *Staphylococcus aureus*, polysaccharides from *Streptococcus agalactae* and *streptococcus pneumoniae* polysaccharides from Mycobacterium, e.g. *Mycobacterium tuberculosis,* such as mannophosphoinisitides trehaloses, mycolic acid, mannose capped arabinomannans, the capsule therefrom and arabinogalactans, polysaccharide from *Cryptococcus neoformans*, the lipopolysaccharides of non-typeable *Haemophilus influenzae*, the capsular polysaccharide from *Haemophilus influenzae b*, the lipopolysaccharides of *Moraxella catharralis*, the lipopolysaccharides of *Shigella sonnei*, the lipopeptidophosphoglycan (LPPG) of *Trypanosoma cruzi*, the cancer associated gangliosides GD3, GD2, the tumor

associated mucins, especially the T-F antigen, and the sialyl T-F antigen, and the HIV associated polysaccharide that is structurally related to the T-F antigen.

The polysaccharide may be linked to the carrier protein by any known method. In particular by CDAP conjugation.

The cyanylating reagent 1-cyano-dimethylaminopyridinium tetrafluoroborate (CDAP) is preferably used for the synthesis of polysaccharide-protein conjugates. CDAP is a water soluble reagent and coupling a higher electrophilic cyano group than CNBr which may also be used in compiling the polysaccharide to the protein. The cyanilation reaction can be performed under relatively mild conditions and avoid hydrolysis of alkaline sensitive polysaccharides. This synthesis allows direct coupling to a carrier protein.

The polysaccharide is solubilized in water or a saline solution. CDAP is dissolved in acetonitrile and added immediately to the polysaccharide solution. The CDAP reacts with the hydroxyl groups of the polysaccharide to form cyanate ester. After the activation step, the carrier protein is added. Amino groups of lysine react with the activated polysaccharide to form an isourea covalent link.

After the coupling reaction, a large excess of glycine is then added to quench residual activated functions. The product is then passed through a gel permeation to remove unreacted carrier protein and residual reagents. Accordingly the invention provides a method of producing polysaccharide protein D conjugates comprising the steps of activating the polysaccharide and linking the polysaccharide to the protein D.

In a preferred embodiment of the invention there is provided a vaccine formulation for the prevention of *Streptococcus pneumoniae* infections.

*Streptococcus pneumoniae* is a gram positive bacteria that is pathogenic for humans, causing invasive diseases such as pneumonia, bacteremia and meningitis, and diseases associated with colonisation, such as acute Otitis media. The mechanisms by which pneumococci spread to the lung, the cerebrospinal fluid and the blood is poorly understood. Growth of bacteria reaching normal lung alveoli is inhibited by their relative dryness and by the phagocytic activity of alveolar macrophages. Any anatomic or physiological changes of these co-ordinated defences tend to augment the susceptibility of the lungs to infection. The cell-wall of

*Streptococcus pneumoniae* has an important role in generating an inflammatory response in the alveoli of the lung (Gillespie et al , I&I 65: 3936).

Typically the *Streptococcus pneumoniae* vaccine of the present invention will comprise protein D polysaccharide conjugates, wherein the polysaccharide are derived from at least four serotypes. Preferably the four serotypes include 6B, 14, 19F and 23F. More preferably, at least 11 serotypes are included in the vaccine, for example the vaccine in one embodiment includes the protein D – capsular polysaccharide conjugate wherein the polysaccharide are derived from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F are included. In a preferred embodiment of the invention at least 13 polysaccharide conjugates are included, although more valents, for example 23 valents are contemplated by the invention.

For elderly vaccination (for the prevention of pneumonia) it is advantageous to include serotypes 8 and 12F to the 11 valent vaccine above. For peadiatric vaccination (where Otitis media is of more concern), it is preferred to include polysaccharide conjugates from serotypes 6A and 19A to the 11 valent vaccines mentioned above.

In a further embodiment of the invention there is provided a Neisseria Meningitidis vaccine; in particular from serotypes A, B, C W-135 and Y. Neisseria Meningitidis is one of the most important causes of bacterial meningitis. The carbohydrate capsule of these organisms can act as a virulence determinant and a target for protective antibody. Carbohydrates are nevertheless well known to be poor immunogens in young children. Their poor immunogenicity is though to be due to the T-cell independent nature of polysaccharide antigens. The absence of a T-cell response prevents B-cell proliferation and maturation, as well as the induction of an immunological memory. The present invention overcomes these limitations by covalently coupling of capsular polysaccharides to protein D carrier which contains T-cell epitopes.

In an alternative embodiment of the invention there is provided a Protein D capsular polysaccharide of *Haemophilus influenzae b* (PRP) conjugate.

The present invention also contemplates combination vaccines which provide protection against a range of different pathogens.

A preferred combination includes a vaccine that affords protection against Neisseria Meningitidis A and C infection wherein the polysaccharide antigen from at least one, and preferably both A and C serotypes are linked to protein D.

Haemophilus influenza polysaccharide based vaccine may be formulated with the above combination vaccines.

Many Paediatric vaccines are now given as a combination vaccine so as to reduce the number of injections a child has to receive. Thus for Paediatric vaccines other antigens may be formulated with the vaccines of the invention. For example the vaccines of the invention can be formulated with, or administered separately, but at the same time with the well known 'trivalent' combination vaccine comprising Diphtheria toxoid (DT), tetanus toxoid (TT), and pertussis components [typically detoxified Pertussis toxoid (PT) and filamentous haemagglutinin (FHA) with optional pertactin (PRN) and/or agglutinin 1+2], for example the marketed vaccine INFANRIX-DTPa™ (SmithKlineBeecham Biologicals) which contains DT, TT, PT, FHA and PRN antigens, or with a whole cell pertussis component for example as marketed by SmithKlineBeecham Biologicals s.a., as Tritanrix™. The combined vaccine may also comprise other antigen, such as Hepatitis B surface antigen (HBsAg), Polio virus antigens (for instance inactivated trivalent polio virus – IPV), *Moraxella catarrhalis* outer membrane proteins, non-typeable *Haemophilus influenzae* proteins, *N.meningitidis* B outer membrane proteins.
Examples of preferred *Moraxella catarrhalis* protein antigens include: OMP106 WO 97/41731 (Antex) & WO 96/34960 (PMC), OMP21, LbpA & LbpB WO 98/55606 (PMC),TbpA & TbpB WO 97/13785 & WO 97/32980 (PMC), CopB Helminen ME, et al (1993) Infect. Immun. 61:2003-2010. UspA1/2 WO93/03761 (University of Texas), and OmpCD. Examples of non-typeable *Haemophilus influenzae* antigens include Fimbrin protein, (US 5766608 - Ohio State Research Foundation) and Fusions comprising peptides therefrom (eg LB1 Fusion) (US 5843464 - Ohio State Research Foundation),OMP26
WO 97/01638 (Cortecs) , P6 EP 281673 (State University of New York), TbpA and TbpB, Hia, Hmw1,2,Hap, and D15.

Preferred Peadiatric vaccines contemplated by the present invention are:

- *N.meningitidis* C polysaccharide – conjugate and *Haemophilus influenzae b* conjugate, optional with *N.meningitidis* A polysaccharide conjugate, provided that at least one polysaccharide antigen, and preferably all are conjugated to protein D.

- *N.meningitidis* C polysaccharide protein D conjugate *Haemophilius influenzae b* PRP conjugate, DT, TT, Pertussis component Hepatitis B surface antigen and I.P.V.

- *Streptococcus pneumoniae* protein D – polysaccharide antigen and one or more antigens from *Moraxella catarrhalis* and/or non-typeable *Haemophilia's influenzae*.

For the prevention of pneumoniae in the elderly (+55 years) population and Otitis media in Infants, it is a preferred embodiment of the invention to combine a multivalent streptococcus pneumonia polysaccharide – protein D antigens as herein described with a *Streptococcus pneumoniae* protein or immunologically functional equivalent thereof. Preferred proteins to be included in such a combination, include but are not limited to, pneumolysin Nucleic Acids Res 1990 Jul 11;18(13):4010 "Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2." Mitchell TJ, Mendez F, Paton JC, Andrew PW, Boulnois GJ , Biochim Biophys Acta 1989 Jan 23;1007(1):67-72 "Expression of the pneumolysin gene in Escherichia coli: rapid purification and biological properties." Mitchell TJ, Walker JA, Saunders FK, Andrew PW, Boulnois GJ. WO 96/05859 (A. Cyanamid), WO 90/06951 (Paton et al) -.WO 99/03884 (NAVA), PspA and transmembrane deletion variants thereof US 5804193 (Briles et al), PspC (WO 97/09994 - Briles et al). PsaA (Infect Immun 1996 Dec;64(12):5255-62 "Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*." Berry AM, Paton JC). Streptococcal choline binding protein (WO97/41151); Glyceraldehyde –3- phosphate – dehydrogenase (I&I 64: 3544), HSP 70 (WO96/40928).

Accordingly the present invention provides an immunogenic composition comprising a *Streptococcus pneumoniae* – polysaccharide – protein D conjugate and a *Streptococcus pneumoniae* protein antigen.

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York). Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

The polysaccharide – protein D conjugate antigens of the present invention are preferably adjuvanted in the vaccine formulation of the invention. Suitable adjuvants include an aluminium salt such as aluminum hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

For Paediatric uses, it is preferred to adjuvant the vaccine with an aluminium salt phosphate. For elderly vaccine it is preferred that the adjuvant be selected to be a preferably aluminium preferential inducer of a TH1 type of response.

High levels of Th1-type cytokines tend to favour the induction of cell mediated immune responses to the given antigen, whilst high levels of Th2-type cytokines tend to favour the induction of humoral immune responses to he antigen.

It is important to remember that the distinction of Th1 and Th2-type immune response is not absolute. In reality an individual will support an immune response which is described as being predominantly Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 +ve T cell clones by Mosmann and Coffman (Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173). Traditionally, Th1-type responses are associated with the production of the INF-$\gamma$ and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2-type responses are associated with the secretion of Il-4, IL-5, IL-6, IL-10. Suitable adjuvant systems include, Monophosphoryl lipid A, particularly 3-de-O-acylated monophosphoryl lipid A, and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salt .

An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, o a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO96/33739.

A particularly potent adjuvant formulation involving QS21 3D-MPL and tocopherol in an oil in water emulsion is described in WO95/17210 and is a preferred formulation.

Preferably the vaccine additionally comprises a saponin, more preferably QS21.

Preferably the formulation additional comprises an oil in water emulsion and tocopherol. The present invention also provides a method for producing a vaccine formulation comprising mixing a protein of the present invention together with a pharmaceutically acceptable excipient, such as 3D-MPL.

Unmethylated CpG containing oligo nucleotides (WO 96/02555) are also a preferential inducer of a TH1 response and are suitable for use in the present invention.

In a further aspect of the present invention there is provided an immunogen or vaccine as herein described for use in medicine.

The vaccine preparation of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts.

The amount of conjugate antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 0.1-100 μg of polysaccharide, preferably 0.1-50 μg, preferably 0.1-10 μg, of which 1 to 5 μg is the most preferable range. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial

vaccination, subjects may receive one or several booster immunisations adequately spaced.

**EXAMPLE I**

**Neisseria Meningitidis C polysaccharide – Protein D conjugate (PSC-PD)**

**A: EXPRESSION OF PROTEIN D**

***Haemophilus influenzae* protein D**

**Genetic construction for protein D expression**

**Starting materials**

***The Protein D encoding DNA***

Protein D is highly conserved among H. influenzae of all serotypes and non-typeable strains. The vector pHIC348 containing the DNA sequence encoding the entire protein D gene has been obtained from Dr. A. Forsgren, Department of Medical Microbiology, University of Lund, Malmö General Hospital, Malmö, Sweden. The DNA sequence of protein D has been published by Janson et al. (1991) I & I *59* : 119-125.

***The expression vector pMG1***

The expression vector pMG1 is a derivative of pBR322, in which bacteriophage λderived control elements for transcription and translation of foreign inserted genes were introduced (Shatzman et al., 1983). In addition, the Ampicillin resistance gene was exchanged with the Kanamycin resistance gene.

***The E. coli strain AR58***

The E. coli strain AR58 was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, λ Kil - cI857 ΔH1). N99 and SA500 are E. coli K12 strains derived from Dr. Martin Rosenberg's laboratory at the National Institute of Health.

***The expression vector pMG 1***

For the production of protein D, the DNA encoding the protein has been cloned into the expression vector pMG 1. This plasmid utilizes signals from λ phage DNA to drive the transcription and translation of inserted foreign genes. The vector

contains the λ promotor PL, operator OL and two utilization sites (NutL and NutR) to relieve transcriptional polarity effects when N protein is provided (Gross et al., 1985). Vectors containing the PL promotor, are introduced into an E. coli lysogenic host to stabilize the plasmid DNA. Lysogenic host strains contain replication-defective λ phage DNA integrated into the genome (Shatzman et al., 1983). The chromosomal λ phage DNA directs the synthesis of the cI repressor protein which binds to the OL repressor of the vector and prevents binding of RNA polymerase to the PL promotor and thereby transcription of the inserted gene. The cI gene of the expression strain AR58 contains a temperature sensitive mutant so that PL directed transcription can be regulated by temperature shift, i.e. an increase in culture temperature inactivates the repressor and synthesis of the foreign protein is initiated. This expression system allows controlled synthesis of foreign proteins especially of those that may be toxic to the cell (Shimataka & Rosenberg, 1981).

***The E. coli strain AR58***

The AR58 lysogenic E. coli strain used for the production of the protein D carrier is a derivative of the standard NIH E. coli K12 strain N99 (F - su - galK2, lacZ - thr - ). It contains a defective lysogenic λ phage (galE::TN10, λ Kil - cI857 ΔH1). The Kil –phenotype prevents the shut off of host macromolecular synthesis. The cI857 mutation confers a temperature sensitive lesion to the cI repressor. The ΔH1 deletion removes the λ phage right operon and the hosts bio, uvr3, and chlA loci. The AR58 strain was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, λ Kil - cI857 ΔH1). The introduction of the defective lysogen into N99 was selected with tetracycline by virtue of the presence of a TN10 transposon coding for tetracyclin resistance in the adjacent galE gene.

***Construction of vector pMGMDPPrD***

The pMG 1 vector which contains the gene encoding the non-structural S1 protein of Influenzae virus (pMGNSI) was used to construct pMGMDPPrD. The protein D gene was amplified by PCR from the pHIC348 vector (Janson et al. 1991) with PCR primers containing NcoI and XbaI restriction sites at the 5' and 3' ends, respectively. The NcoI/XbaI fragment was then introduced into pMGNS1 between NcoI and XbaI thus creating a fusion protein containing the N-terminal 81 amino

acids of the NS1 protein followed by the PD protein. This vector was labeled pMGNS1PrD.

Based on the construct described above the final construct for protein D expression was generated. A BamHI/BamHI fragment was removed from pMGNS1PrD. This DNA hydrolysis removes the NS1 coding region, except for the first three N-terminal residues. Upon religation of the vector a gene encoding a fusion protein with the following N-terminal amino acid sequence has been generated:

-----MDP SSHSSNMANT-----

NS1 Protein D

The protein D does not contain a leader peptide or the N-terminal cysteine to which lipid chains are normally attached. The protein is therefore neither excreted into the periplasm nor lipidated and remains in the cytoplasm in a soluble form.

The final construct pMG-MDPPrD was introduced into the AR58 host strain by heat shock at 37°C. Plasmid containing bacteria were selected in the presence of Kanamycin. Presence of the protein D encoding DNA insert was demonstrated by digestion of isolated plasmid DNA with selected endonucleases. The recombinant E. coli strain is referred to as ECD4.

Expression of protein D is under the control of the $\lambda$ $P_L$ promotor/ $O_L$ Operator. The host strain AR58 contains a temperature-sensitive cI gene in the genome which blocks expression from $\lambda$ $P_L$ at low temperature by binding to $O_L$. Once the temperature is elevated cI is released from $O_L$ and protein D is expressed. At the end of the fermentation the cells are concentrated and frozen.

The extraction from harvested cells and the purification of protein D is described below:

The frozen cell culture pellet is thawed and resuspended in a cell disruption solution (Citrate buffer pH 6.0) to a final $OD_{650}$ = 60. The suspension is passed twice through a high pressure homogenizer at P = 1000 bar. The cell culture homogenate is clarified by centrifugation and cell debris are removed by filtration. In the first purification step the filtered lysate is applied to a cation exchange chromatography column (SP Sepharose Fast Flow). PD binds to the gel matrix by ionic interaction and is eluted by a step increase of the ionic strength of the elution buffer.

In a second purification step impurities are retained on an anionic exchange matrix (Q Sepharose Fast Flow). PD does not bind onto the gel and can be collected in the flow through.

In both column chromatography steps fraction collection is monitored by OD. The flow through of the anionic exchange column chromatography containing the purified protein D is concentrated by ultrafiltration.

The protein D containing ultrafiltration retentate is finally passed through a 0.2 µm membrane.

## B: MANUFACTURE OF POLYSACCHARIDE C

The source of group C polysaccharide is the strain C11 of N. meningitidis. This is fermented using classical fermentation techniques (EP 72513). The dry powder polysaccharides used in the conjugation process are identical to Mencevax (SB Biologicals s.a.).

*Solid pre-cultures*

An aliquot of C11 strain is thawed and 0.1ml of suspension is streaked on one Mueller Hinton medium petri dish supplemented with yeast extract dialysate (10%, v/v) and incubated for 23 to 25hrs at 36°C in a water saturated air incubator.

The surface growth is then re-suspended in sterilized fermentation medium and inoculated with this suspension on one Roux bottle containing Mueller Hinton

medium supplemented with yeast extract dialysate (10%, v/v) and sterile glass beads. After incubation of the Roux bottle during 23 to 25hrs at 36°C in a water saturated air incubator, the surface growth is re-suspended in 10ml sterile fermentation medium and 0.2 to 0.3ml of this suspension are inoculated onto 12 other Mueller Hinton medium Roux bottles.

After incubation during 23 to 25hrs at 36°C in a water saturated air incubator, surface growth is re-suspended in 10ml sterile fermentation medium. The bacterial suspension is pooled in a conical flask.

This suspension is then aseptically transferred into the fermenter using sterile syringes.

***Fermentation***

The fermentation of meningococcus is performed in fermenters contained in a clean room under negative pressure. The fermentation is generally completed after 10-12hrs corresponding to approximately $10^{10}$ bacteria/ml (i.e. the early stationary phase) and detected by pH increase.

At this stage, the entire broth is heat inactivated (12 min at 56°C) before centrifugation. Before and after inactivation, a sample of the broth is taken and streaked onto Mueller Hinton medium petri dishes.

## C: PS PURIFICATION

The purification process is a multi-step procedure performed on the entire fermentation broth. In the first stage of purification, the inactivated culture is clarified by centrifugation and the supernatant is recovered.

Polysaccharides purification is based on precipitation with a quaternary ammonium salt (Cetyltrimethylammonium Bromide/CTAB,CETAVLON R ). CTAB forms insoluble complexes with polyanions such as polysaccharides, nucleic acid and

proteins depending on their pI. Following ionic controlled conditions, this method can be used to precipitate impurities (low conductivity) or polysaccharides (high conductivity).

The polysaccharides included in clarified supernatant are precipitated using a diatomaceous earth (CELITE$^{R}$ 545) as matrix to avoid formation of insoluble inert mass during the different precipitations/purifications.

***Purification scheme for N. meningitidis polysaccharide C:***

**Step1**: PSC-CTAB complex fixation on CELITE$^{R}$ 545 and removal of cells debris, nucleic acids and proteins by washing with CTAB 0.05%.

**Step 2**: Elution of PS with EtOH 50%. The first fractions which are turbid and contain impurities and LPS are discarded. The presence of PS in the following fractions is verified by floculation test.

**Step3**: PS-CTAB complex re-fixation on CELITE R 545 and removal of smaller nucleic acids and proteins by CTAB 0.05% washing.

**Step 4**: Elution of PS with EtOH 50%. The first turbid fractions are discarded. The presence of PS in the following fractions is verified by floculation test.

The eluate is filtered and the filtrate containing crude polysaccharide collected.

The polysaccharide is precipitated from the filtrate by adding ethanol to a final concentration of 80 %.

The polysaccharide is then recovered as a white powder, vaccum dried and stored at -20°C.

## D: CDAP CONJUGATION

*Conjugation of PSC and PD*

For conjugation of PSC and PD, the CDAP conjugation technology was preferred to the classical CNBr activation and coupling via a spacer to the carrier protein. The polysaccharide is first activated by cyanylation with 1-cyano-4-dimethylamino-

pyridinium tetrafluoroborate (CDAP). CDAP is a water soluble cyanylating reagent in which the electrophilicity of the cyano group is increased over that of CNBr, permitting the cyanylation reaction to be performed under relatively mild conditions. After activation, the polysaccharide can be directly coupled to the carrier protein through its amino groups without introducing any spacer molecule. The unreacted estercyanate groups are quenched by means of extensive reaction with glycine. The total number of steps involved in the preparation of conjugate vaccines is reduced and most importantly potentially immunogenic spacer molecules are not present in the final product.

Activation of polysaccharides with CDAP introduces a cyanate group in the polysaccharides and dimethylaminopyridine (DMAP) is liberated. The cyanate group reacts with NH2-groups in the protein during the subsequent coupling procedure and is converted to a carbamate.

***PSC activation and PSC-PD coupling***

Activation and coupling are performed at +25°C.

120 mg of PS is dissolved for at least 4h in WFI.

CDAP solution (100 mg/ml freshly prepared in acetonitrile) is added to reach a CDAP/PS (w/w) ratio of 0.75.

After 1 min 30, the pH is raised up to activation pH (pH 10) by addition of triethylamine and is stabilised up to PD addition.

At time 3 min 30, NaCl is added to a final concentration of 2M.

At time 4 min, purified PD is added to reach a PD/PS ratio of 1.5/1; pH is immediately adjusted to coupling pH (pH 10). The solution is left for 1h under pH regulation.

***Quenching***

6 ml of a 2M glycine solution is added to the PS/PD/CDAP mixture. The pH is adjusted to the quenching pH (pH 8.8). The solution is stirred for 30 min at the working T°, then overnight at +2-8°C with continuous slow stirring.

*PS-PD purification*

After filtration (5 µm), the PS-PD conjugate is purified in a cold room by gel permeation chromatography on a S400HR Sephacryl gel to remove small molecules (including DMAP) and unconjugated PD:

- Elution: NaCl 150 mM pH 6.5.
- Monitoring: UV 280 nm, pH and conductivity.

Based on the different molecular size of the reaction components, PS-PD conjugates are eluted first followed by free PD and finally DMAP. Fractions containing conjugate as detected by DMAB (PS) and µBCA (protein) are pooled. The pooled fractions are sterile filtered (0.2 µm)

## E: FORMULATION OF PSC-PD ADSORBED CONJUGATE VACCINE

*Washing of $AlPO_4$*

In order to optimize the adsorption of PSC-PD conjugate on $AlPO_4$, the $AlPO_4$ is washed to reduce the $PO_4^{3-}$ concentration:

- AlPO4 is washed with NaCl 150 mM and centrifuged (4x),
- the pellet is then resuspended in NaCl 150 mM,
- filtrated (100 µm) and
- the filtrate is heat sterilized

This washed $AlPO_4$ is referred to as WAP (washed autoclaved phosphate).

*Formulation process*

The PSC-PD conjugate bulk is adsorbed on AlPO4 WAP before the final formulation of the finished product. Formulation of PSC-PD adsorbed conjugate vaccine is shown in Figure 1.

*Final composition/dose*

| | |
|---|---|
| - PSC-PD: | 10 µg PS |
| - AlPO4 WAP: | 0.25 mg Al 3+ |
| - NaCl: | 150 mM |
| - 2-phenoxy-ethanol: | 2.5 mg |
| - Water for Injection: | to 0.5 ml |
| - pH: | 6.1 |

## F: PRECLINICAL INFORMATION

*Immunogenicity of polysaccharide conjugate in mice*

The immunogenicity of the PSC-PD conjugate has been assessed in 6- to 8-weeks-old Balb/C mice. The plain (unadsorbed) conjugate or the conjugate adsorbed onto AlPO4 was injected as a monovalent vaccine. Anti-PSC antibodies induced were measured by ELISA whilst functional antibodies were analysed using the bactericidal test, both methods being based on the CDC (Centers for Disease Control and Prevention, Atlanta, USA) protocols. Results from two different experiments performed to assess the response versus the dose and adjuvant ($AlPO_4$) effect are presented.

*Dose-range experiment*

In this experiment, the PSC-PD was injected twice (two weeks apart) in Balb/C mice. Four different doses of conjugate formulated on AlPO4 were used: 0.1 - 0.5 - 2.5 and 9.6 µg/animal. The mice (10/group) were bled on days 14 (14 Post I), 28 (14 Post II) and 42 (28 Post II). Geometric mean concentrations (GMCs) of polysaccharide C specific antibodies measured by ELISA were expressed in µg IgG/ml using purified IgG as reference Bactericidal antibodies were measured on pooled sera and titres expressed as the reciprocal of the dilution able to kill 50 % of bacteria, using the N. meningitidis C11 strain in presence of baby rabbit complement.

The dose-response obtained show a plateau from the dose of 2.5 µg. Results indicate that there is a good booster response between 14 Post I and 14 Post II. Antibody levels at 28 Post II are at least equivalent to those at 14 Post II. Bactericidal antibody titres are concordant with ELISA concentrations and confirm the immunogenicity of the PSC-PD conjugate.

*Effect of adjuvant*

In this experiment, one lot of PSC-PD conjugate formulated on AlPO4 was assessed, the plain (non-adjuvanted) conjugate was injected for comparison. 10 mice/group were injected twice, two weeks apart, by the subcutaneous route, with 2

µg of conjugate. Mice were bled on days 14 (14 Post I), 28 (14 Post II) and 42 (28 Post II), and ELISA (Figure 28) and functional antibody titres measured (only on 14 Post II and 28 Post II for the bactericidal test). The AlPO4 formulation induces up to 10 times higher antibody titres as compared to the non-adjuvanted formulations.

*Conclusions*

The following general conclusions can be made from the results of the experiments described above:

- PSC-PD conjugate induces an anamnestic response demonstrating that PSC, when conjugated, becomes a T cell dependent antigen.
- Anti-PSC antibody concentrations measured by ELISA correlate well with bactericidal antibody titres showing that antibodies induced by the PSC-PD conjugate are functional against N. meningitidis serogroup C.
- Approximately 2.5 µg of conjugate adsorbed onto AlPO4 appears to elicit an optimum antibody response in mice.
- The CDAP chemistry appears to be a suitable method for making immunogenic PSC-PD conjugates.

**EXAMPLE 2**

**PREPARATION OF A POLYSACCHARIDE FROM NEISSERIA MENINGITIDIS SEROGROUP A - PD CONJUGATE**

A dry powder of polysaccharide A (PS A) is dissolved during one hour in NaCl 0.2 M solution to a final concentration of 8 mg/ml. pH is then fixed to a value of 6 with either HCl or NaOH and solution thermoregulated at 25°C. 0.75 mg CDAP/mg PSA (a preparation to 100 mg/ml acetonitrile) is added to the PSA solution. After 1.5 minute without pH regulation, NaOH 0.2 M is added to obtain a pH of 10. 2.5 minutes later, protein D (concentrated to 5 mg/ml) is added according to a PD/PS A ratio of approximately 1. A pH of 10 is maintained during the coupling reaction period of 1 hour. Then, 10 mg glycine (2 M pH 9.0)/mg PS A is added and pH regulated at a value of 9.0 during 30 minutes at 25°C. The mixture is then conserved overnight at 4°C before purification by an exclusion column

chromatography (Sephacryl S400HR from Pharmacia). The conjugate elutes first followed by unreacted PD and by-product (DMAP, glycine, salts). The conjugate is collected and sterilized by a 0.2 $\mu$m filtration on a Sartopore membrane from Sartorius.

**EXAMPLE 3**

**IN VITRO CHARACTERISATIONS**

The major characteristics are summarized in the table here below.

| N° | Conjugate description | Protein and PS content ($\mu$g/ml) | PS/protein ratio (w/w) | Free Protein (%) | Free PS (%) |
|---|---|---|---|---|---|
| 1 | PS C - PD NaOH for pH regulation | PD : 210 PS : 308 | 1/0.68 | < 2 | 8-9 |
| 2 | PS C - PD TEA for pH regulation | PD : 230 PS : 351 | 1/0.65 | < 2 | 5-6 |
| 3 | PS A - PD NaOH for pH regulation | PD : 159 PS : 149 | 1/1.07 | 5 | 5-9 |

***In vivo results***

Balb/C mice were used as animal model to test the immunogenicity of the conjugates. The conjugates were adsorbed either onto $AlPO_4$ or $Al(OH)_3$ (10 $\mu$g of PS onto 500 $\mu$g of $Al^{3+}$) or not adsorbed. The mice were injected as followed : 2 injections at two week intervals (2 $\mu$g PS/injection).

From these results, we can conclude first that free PS influence greatly the immune response. Better results have been obtained with conjugates having less than 10 % free PS.

The formulation is also important. $AlPO_4$ appears to be the most appropriate adjuvant in this model.

The conjugates induce a boost effect which is not observed when polysaccharides are injected alone.

*Conclusions*

Conjugates of N. Meningitidis A and C were obtained with a final PS/protein ratio of 1 and 0.6-0.7 (w/w) respectively.

Free PS and free carrier protein were below 10 % and 15 % respectively.

Polysaccharide recovery is higher than 70 %.

**EXAMPLE 4 VACCINE COMPONENTS:**

*S.pneumoniae capsular polysaccharide:*

The 11-valent candidate vaccine includes the capsular polysaccharides serotypes 1,3,4,5,6B, 7F, 9V,14,18C,19F and 23F which were made essentially as described in EP72513.

Each polysaccharide is activated and derivatized using the CDAP chemistry and conjugated to the protein carrier.

All the polysaccharides are conjugated in their native form,except for the serotype 3.Its size was reduced by micro-fluidization.

***Protein carrier:***

The protein carrier selected is the recombinant protein D (PD) from Non typeable *Haemophilus influenzae*, expressed in E.coli.

***Chemistry:***

***Activation and coupling chemistry:***

The activation and coupling conditions are specific for each polysaccharide .These are given in Table 1.

Native polysaccharide (except for PS3) was dissolved in NaCl 2M or in water for injection. The optimal polysaccharide concentration was evaluated for all the serotypes.

From a 100 mg/ml stock solution in acetonitrile ,CDAP (CDAP/PS ratio:0.75 mg/mg PS) was added to the polysaccharide solution.1.5 minute later,0.2M triethylamine was added to obtain the specific activation pH. The activation of the polysaccharide was performed at this pH during 2minutes at 25°C.Protein D (the quantity depends on the initial PS/PD ratio) was added to the activated polysaccharide and the coupling reaction was performed at the specific pH for 1 hour.

Then, the reaction was quenched with glycine for 30 minutes at 25°C and overnight at 4°C.

The conjugates were purified by gel filtration using a Sephacryl 500HR gel filtration column equilibrated with 0.2M NaCl.

The carbohydrate and protein contents of the eluted fractions were determined .The conjugates were pooled and sterile filtered on a 0.22$\mu$m sterilizing membrane. The PS/Protein ratios in the conjugate preparations were determined.

***Characterization:***

Each conjugate was characterized and meet the specifications described in Table 2.

**Polysaccharide and protein content ($\mu$g/ml):**

The polysaccharide content was measured by the Resorcinol test and the protein content by the Lowry test. The final PS/PD ratio(w/w) is determined by the ratio of the concentrations.

***Residual DMAP content (ng/$\mu$g PS):***

The activation of the polysaccharide with CDAP introduces a cyanate group in the polysaccharide and DMAP (4-dimethylamino-pyridin) is liberated. The residual DMAP content was determined by a specific assay developed and validated at SB.

**Free polysaccharide content (%):**

The free polysaccharide content on conjugates kept at 4°C or stored 7 days at 37°C was determined on the supernatant obtained after incubation with α-PD antibodies and saturated ammonium sulfate, followed by a centrifugation.

An α-PS/α-PS ELISA was used for the quantification of free polysaccharide in the supernatant . The absence of conjugate was also controlled by an α-PD/α-PS ELISA.

***Antigenicity:***

The antigenicity on the same conjugates was analyzed in a sandwich-type ELISA wherein the capture and the detection of antibodies were α-PS and α-PD respectively.

***Free protein content (%):***

The level of "free" residual protein D was determined by using a method with SDS treatment of the sample. The conjugate was heated 10 min at 100°C in presence of SDS 0.1 % and injected on a SEC-HPLC gel filtration column (TSK 3000-PWXL).As protein D is dimmer, there is a risk to overestimate the level of "free" protein D by dissociation the structure with SDS.

***Molecular size ($K_{av}$):***

The molecular size was performed on a SEC-HPLC gel filtration column (TSK 5000-PWXL).

***Stability:***

The stability was performed on a HPLC-SEC gel filtration (TSK 6000-PWXL) on conjugates kept at 4°C and stored for 7 days at 37°C.

The 11-valent characterization is given in Table 2

The protein conjugates can be adsorbed onto aluminium phosphate and pooled to form the final vaccine.

**Figure 1 : Formulation of PSC-PD adsorbed conjugate vaccine**

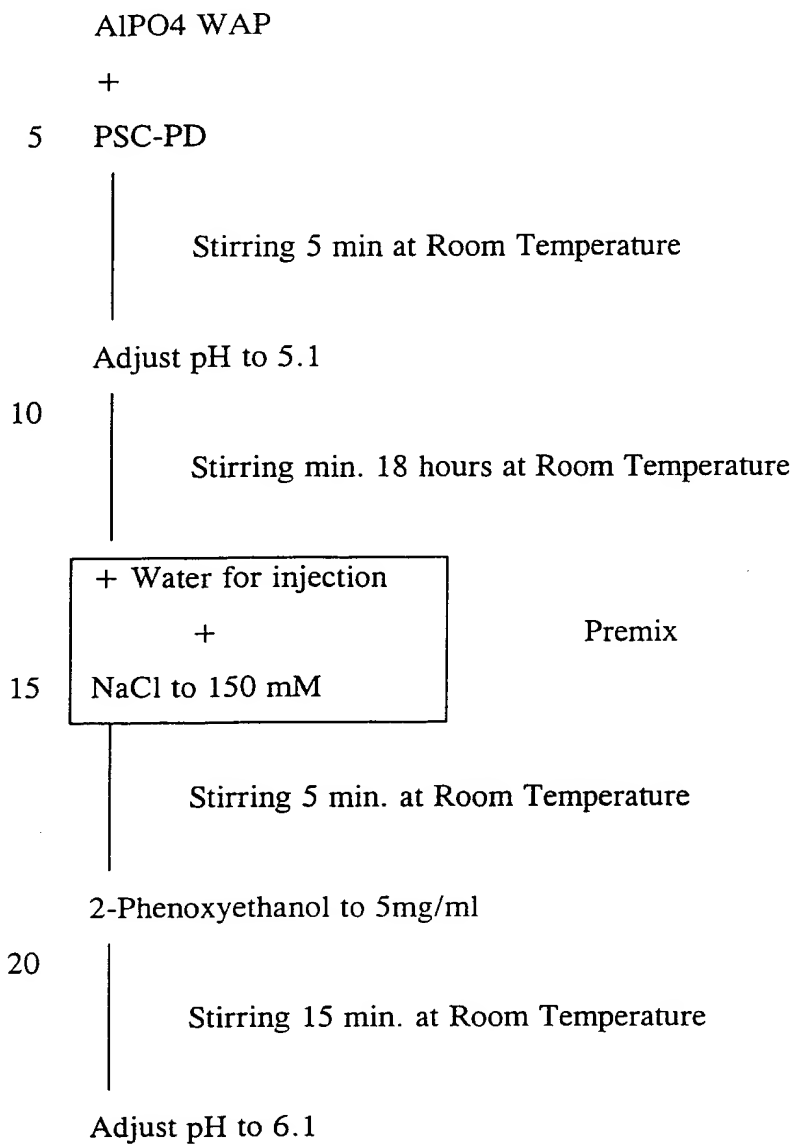

Table 1

Specific activation/coupling/quenching conditions of PS S.pneumoniae-Protein D conjugates

| Serotype | 1 | 3 ($\mu$fluid.) | 4 | 5 | 6B | 7F |
|---|---|---|---|---|---|---|
| PS conc.(mg/ml) | 2.0 | 3.0 | 2.0 | 7.5 | 5.4 | 3.0 |
| PS dissolution | NaCl 2M | NaCl 2M | $H_2O$ | $H_2O$ | NaCl 2M | NaCl 2M |
| PD conc.(mg/ml) | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| Initial PS/PD Ratio (w/w) | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 |
| CDAP conc. (mg/mg PS) | 0.75 | 0.75 | 0.75 | 0.75 | 0.75 | 0.75 |
| $pH_a = pH_c = pH_q$ | 9.0/9.0/9.0 | 9.0/9.0/9.0 | 9.0/9.0/9.0 | 9.0/9.0/9.0 | 9.5/9.5/9.0 | 9.0/9.0/9.0 |

| Serotype | 9V | 14 | 18C | 19F | 23F |
|---|---|---|---|---|---|
| PS conc.(mg/ml) | 2.5 | 2.5 | 2.0 | 4.0 | 3.3 |
| PS dissolution | NaCl 2M | NaCl 2M | $H_2O$ | NaCl 2M | NaCl 2M |
| PD conc.(mg/ml) | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| Initial PS/PD Ratio (w/w) | 1/0.75 | 1/0.75 | 1/1 | 1/0.5 | 1/1 |
| CDAP conc. (mg/mg PS) | 0.75 | 0.75 | 0.75 | 0.75 | 0.75 |
| $pH_a = pH_c = pH_q$ | 8.5/8.5/9.0 | 9.0/9.0/9.0 | 9.0/9.0/9.0 | 10/9.5/9.0 | 9.0/9.0/9.0 |

TABLE 2

| Criteria | D01PDJ227 | D03PDJ236 | D4PDJ228 | D5PDJ235 | D6PDJ209 |
|---|---|---|---|---|---|
| Ratio PS/Prot (w/w) | 1/0.66 | 1/1.09 | 1/0.86 | 1/0.86 | 1/0.69 |
| Free polysac. content (%) <10 % | 1 | 1 | 7 | 9 | 0 |
| Free protein content (%) <15 % | 8 | <1 | 19 | 21 | 9 |
| DMAP content (ng/μg PS) < 0.5 ng/μg PS | 0.2 | 0.6 | 0.4 | 1.2 | 0.3 |
| Molecular size ($K_{av}$) | 0.18 | 0.13 | 0.12 | 0.11 | 0.13 |
| Stability | no shift | no shift | no shift | low shift | no shift |

| | D07PDJ225 | D09PDJ222 | D14PDJ202 | D18PDJ221 | D19PDJ206 | D23PDJ212 |
|---|---|---|---|---|---|---|
| Ratio PS/Prot (w/w) | 1/0.58 | 1/0.80 | 1/0.68 | 1/0.62 | 1/0.45 | 1/0.74 |
| Free polysac. content (%) <10 % | 1 | <1 | <1 | 4 | 4 | 0 |
| Free protein content (%) <15 % | 8 | 0.3 | 3 | 21 | 10 | 12 |
| DMAP content (ng/μg PS) <0.5 ng/μg PS | 0.1 | 0.6 | 0.3 | 0.2 | 0.1 | 0.9 |
| Molecular size ($K_{av}$) | 0.14 | 0.14 | 0.17 | 0.10 | 0.12 | 0.12 |
| Stability | no shift | no shift | no shift | no shift | shift | no shift |

## Claims

1. A polysaccharide conjugate antigen comprising a polysaccharide antigen derived from a pathogenic bacterium conjugated to protein D from *Haemophilus influenzae* or a fragment thereof.

2. A polysaccharide conjugate as claimed in claim 1 wherein the polysaccharide antigens are selected from the Vi polysaccharide from *Salmonella typhi*, meningococcal polysaccharides including type A, C, W135 and Y), the polysaccharide and modified polysaccharides of group B meningococcus, polysaccharides from *Staphylococcus aureus*, polysaccharides from *Streptococcus agalactae* and *Streptococcus pneumoniae,* polysaccharides from Mycobacteria, [eg *Mycobacterium tuberculosis,* such as mannophosphoinisitides trehaloses, mycolic acid, mannose capped arabinomannans], the capsule therefrom and arabinogalactans, polysaccharide from *Cryptococcus neoformans*, the lipopolysaccharides of non-typeable *Haemophilus influenzae*, the capsular polysaccaharide from *Haemophilus influenzae b*, the lipopolysaccharides of *Moraxella catharralis*, the lipopolysaccharides of *Shigella sonnei*, the lipopeptidophosphoglycan (LPPG) of *Trypanosoma cruzi*, the cancer associated gangliosides GD3, GD2, the tumor associated mucins, especially the T-F antigen, and the sialyl T-F antigen, and the HIV associated polysaccharide that is structurally related to the T-F antigen.

3. An immunogenic composition comprising a plurality of polysaccharide conjugate antigens as claimed in claim 1 or 2.

4. An immunogenic composition as claimed in claim 3 comprising *Streptococcus pneumoniae* polysaccharide antigens from at least four *Streptococcus pneumoniae* serotypes.

5. An immunogenic composition as claimed in claim 4, wherein the polysaccharide antigens are derived from serotypes 6B, 14, 19F and 23F.

6. An immunogenic composition as claimed in any one of claims 3 to 5 comprising at least 11 polysaccharide antigen-conjugates derived from different streptococcus peneumoniae serotypes.

7. An immunogenic composition as claimed in any of claims 3 to 6 additionally comprising a *Streptococcus pneumoniae* protein.

8. An immunogenic composition comprising a protein D – Neisseria Meningitidis polysaccharide antigen conjugate.

9. An immunogenic composition as claimed in claim 8 wherein the polysaccharide antigen is derived from serotype A or C or a combination thereof.

10. An immunogenic composition comprising a protein D – *Haemophilius influenzae b* polysaccharide antigen conjugate.

11. An immunogenic composition as claimed in any of claims 8 to 10 wherein the polysaccharide antigens include antigens derived from Neisseria Meningitidis and *Haemophilius influenzae b* antigen.

12. An immunogenic composition as claimed herein additionally comprising an adjuvant.

13. An immunogenic composition as claimed in claim 12 wherein the polysaccharide – protein D conjugate is adsorbed onto aluminium phosphate.

14. An immunogenic composition as claimed in claim 13 wherein the adjuvant is a preferential inducer of a TH1 type of response.

15. An immunogenic composition as claimed herein for use in medicine.

16. A method of producing a polysaccharide – protein D conjugate, comprising activating said polysaccharide and coupling it to protein D.

17. A method of treating a patient suffering from or susceptible to infection from a pathogen bacterium comprising administering an effective amount of an immunogenic composition as claimed herein.